Journal of Microbiology and Biotechnology
Vol. 3, No. 3, 181-187, 1993

Purification of a Steroid Δ^1-dehydrogenase from *Arthrobacter simplex*

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Steroid Δ^1-dehydrogenase which introduces a double bond into the 1,2 positions of steroid ring A was purified from *Arthrobacter simplex*, an excellent biotransformer of hydrocortisone into prednisolone. Hydrocortisone-induced cells were disrupted by vigorous agitation with glass beads, and a solubilized enzyme was obtained after centrifugation at 100,000×*g* for 90 minutes. The enzyme was purified 123-fold in three steps of chromatographic procedures with 13% yield. The last step of testosterone-agarose affinity column decisively contributed to the successful purification. The molecular weight of the enzyme was estimated to be 98,000 by SDS-PAGE and 100,000 by gel filtration, indicating that this enzyme behaves as a monomer. The enzyme showed demands for artificial electron acceptor, and among the several reagents tested, phenazine methosulfate acted as the most effective electron acceptor. Subcellular distribution of this enzyme was studied by centrifugation experiment. Comparison of the enzyme activities in pelleted membrane and cytosol fractions suggests that the enzyme may be a weakly attached peripheral membrane protein *in vivo*. But considerable amounts of enzyme was solubilized without any additional treatments for membrane protein.

It has been known that many microorganisms, including Gram-positive, Gram-negative bacteria and fungi, have abilities to degrade and utilize sterols as carbon source (18, 21, 25). Because steroid Δ^1-dehydrogenation, which introduces 1,2 double bond into steroid ring A (Fig. 1), is one of the critical steps of steroid ring degradation (15, 16, 22), steroid degrading microorganisms usually show Δ^1-dehydrogenase activity. From the first discovery in 1950s (5) microorganisms harboring steroid Δ^1-dehydrogenase have been used in the production of corticosteroid drugs (4, 11, 12, 20, 23, 26). In spite of the long years of industrial applications, there has been little understanding about the enzyme itself. In many species of the genera *Pseudomonas, Mycobacterium, Arthrobacter, Nocardia,* and *Bacillus*, steroid Δ^1-dehydrogenase has been known to be inducible with steroids (15, 27) and membrane bound (14, 16, 19, 28). Recently a soluble flavoprotein catalyzing dehydrogenation of C_1-C_2 bond of 3-ketosteroid has been purified and characterized from *Nocardia corallina* (8, 9).

Arthrobacter simplex which can degrade various steroids and steroids has been used in the microbial transformation of hydrocortisone into Δ^1-dehydrogenated product, prednisolone, as free cells in fermentation broth or immobilized cells (11, 23). The optimum conditions for enzyme induction of *Arthrobacter simplex* has been studied (2).

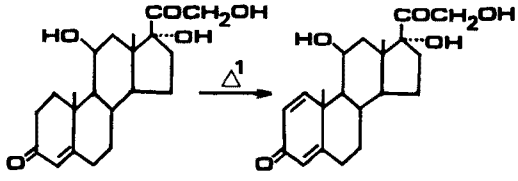

Fig. 1. Δ^1-Dehydrogenation of hydrocortisone into prednisolone.

In this paper we report the purification and some characterization of the steroid Δ^1-dehydrogenase from *Arthrobacter simplex*.

MATERIALS AND METHODS

Chemicals

Hydrocortisone, DEAE-cellulose, phenyl-sepharose,

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Key words: *Arthrobacter simplex*, Steroid Δ^1-dehydrogenase, purification

testosterone-agarose, phenazine methosulfate, and nitroblue tetrazolium were purchased from Sigma. Kieselgel 60 GF_{254} was purchased from Merck, and Sephacryl S-200 was from Pharmacia.

Bacterial Strain and Cell Growth

Arthrobacter simplex IAM 1660 was grown in a medium containing 0.1% NH_4NO_3, 0.025% $MgSO_4 \cdot 7H_2O$, 0.025% K_2HPO_4, 0.5% yeast extract and 0.05% Tween 80 (pH 7.3), with vigorous shaking at 30℃. Hydrocortisone dissolved in ethanol was added to the 14 hour-old culture at the final concentration of 0.01%. After 18 hours of further shaking, cells were harvested.

Determination of Enzyme Activity

Steroid Δ^1-dehydrogenase activity was assayed by measuring the quantity of prednisolone, the reaction product of hydrocortisone, on the silica gel thin layer chromatogram using a densitometer (DESAGA CD60, Heidelberg). An appropriate amount of enzyme solution was added to the reaction mixture containing 0.2 ml of 50 mM Tris buffer (pH 9.0), 20 μg of hydrocortisone in 20 μl of ethanol, and 20 μg of phenazine methosulfate (PMS) in 20 μl of distilled water, and the reaction was carried out in dark at 30℃ for a given period. Steroids were extracted three times with two volumes of ethyl acetate and the organic phase was evaporated to dryness. The residues were dissolved in ethanol and spotted to the silica gel plates prepared with Kieselgel 60 GF_{254}. After developing with benzene-dioxane (2 : 1, v/v), the amount of steroids was measured by a densitometer at 242 nm. One unit of the enzyme was defined as the amount of enzyme producing 1 μmole of prednisolone per minute.

Cell Disruption and Fractionation of Cell Membrane and Cytosol Portions

Grown cells were harvested and washed twice with cold 50 mM potassium phosphate buffer (pH 7.5). After several times of freeze and thaw cycles, cells were resuspended in a small volume of the same buffer containing 5% glycerol. Cells were disrupted by vigorous stirring with the same volume of glass beads in the bead beating cell disrupter (Biospec Product) equipped with an ice jacket preventing heat production. The beating was regularly interrupted to minimize overheating (eg. 30 sec of beating followed by 1 min of intermission). Undisrupted cells and glass beads were removed by centrifugation at 10,000×*g* for 15 minutes. The supernatant was subjected to ultracentrifugation at 100,000×*g* for 90 minutes. The pellet was resuspended in 10 mM potassium phosphate (pH 7.5) containing 20% glycerol (buffer A). Enzyme activities of the pellet and the supernatant were determined.

Purification of Steroid Δ^1-Dehydrogenase

To the 100,000×*g* supernatant obtained from 50 g wet weight cells, streptomycin sulfate was added to the concentration of 0.7%, followed by stirring at 0℃ for 1 hour, and precipitated nucleic acids were removed by centrifugation. The solubilized steroid Δ^1-dehydrogenase preperation was loaded onto the DEAE-cellulose column (2.6×12.5 cm) equilibrated with buffer A containing 20 mM potassium chloride. The column was washed with the same buffer and eluted with increasing gradient of 1.2 *l* of 20 mM~1 M KCl in buffer A. The active fractions were collected, concentrated by ultrafiltration using Amicon stirred cell, dialyzed against buffer A containing 2 M KCl, and loaded onto the phenyl-sepharose column(1.6×8.0 cm) equilibrated with buffer A containing 2 M KCl. After washing with buffer mentioned above, the column was eluted with decreasing gradient of 600 ml of 2~0 M KCl. The pooled active fractions were concentrated, dialyzed against buffer A, and applied to the testosterone-agarose column (2×3 cm) equilibrated with buffer A. After being washed with buffer A, the testosterone-agarose column was eluted with increasing gradient of 400 ml of 0~500 mM phosphate. All the procedures were carried out at 4℃.

Determination of Protein Concentrations

Protein concentrations were measured by the method of Lowry *et al.* (17) using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (13) with 10% running gel. Native gel electrophoresis was performed without SDS and mercaptoethanol. Proteins were stained with coomassie brilliant blue R-250. Activity staining of steroid Δ^1-dehydrogenase in native gel was carried out using oxidoreductase specific dye, nitroblue tetrazolium (NBT) (7), in 50 mM Tris buffer (pH 9.0) containing hydrocortisone and phenazine methosulfate.

Determination of Molecular Weight of the Enzyme

The molecular weight of native enzyme was estimated by gel filtration on Sephacryl S-200 column (1×100 cm). The molecular marker proteins were β-amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), and carbonic anhydrase (29,000). SDS-PAGE was performed to estimate the molecular weight of the denatured polypeptide. The markers for SDS-PAGE were β-galactosidase (116,000), phosphorylase b (97,400), bovine albumin (66,000), egg albumin (45,000), and carbonic anhydrase (29,000).

RESULTS

Cell Disruption and Enzyme Solubilization

Because the pellet of *Arthrobater simplex* cells was

Table 1. Comparison of steroid Δ^1-dehydrogenase activities released by two different cell disruption methods

Cell disruption methods	Enzyme activity* ($U\times10^3$)	Protein (mg)	Specific activity ($U\times10^3$/mg protein)
Sonication	48.6	7.32	6.64
Agitation with glass beads	152.6	11.30	13.50

*Enzyme activities were assayed with crude extracts from 0.5 g wet weight cells

gummy, possibly due to the contents of the cell wall, cell disruption by ultrasonication did not bring satisfactory results. Cell disruption by vigorous agitation with glass beads (0.1 mm in dia.) was more suitable for cell lysis and solubilization of steroid Δ^1-dehydrogenase in *Arthrobater simplex* (Table 1).

Purification of Steroid Δ^1-Dehydrogenase

Steroid Δ^1-dehydrogenase was purified by ion exchange, hydrophobic, and affinity column chromatographic procedures. Solubilized enzyme preparation was applied to the DEAE-cellulose column and active fractions were eluted at 0.2~0.3 M of KCl gradient (Fig. 2). Eluate of the fraction number 55 to 85 were pooled, concentrated, dialyzed and applied to the phenyl-sepharose column. Active fractions were eluted at 0.9~0 M of KCl (Fig. 3). Fration 61 to 79 were pooled, concentrated, dialyzed and applied to the testosterone-agarose column. Active fractions were eluted at 50~100 mM of phosphate (fraction 32 to 42) (Fig. 4). The overall purification procedures are summarized in Table 2. Steroid Δ^1-dehydrogenase was purified 123-fold in three steps with 13% yield.

Non-denaturing PAGE of the purified enzyme revealed a single band with coomassie blue staining (Fig. 5b). Activity staining with nitroblue tetrazolium in the reaction buffer containing hydrocortisone and phenazine methosulfate also showed a single band of violet color at the same position, indicating the dehydrogenation of hydrocortisone, and the presence of steroid Δ^1-dehydrogenase (Fig. 5a).

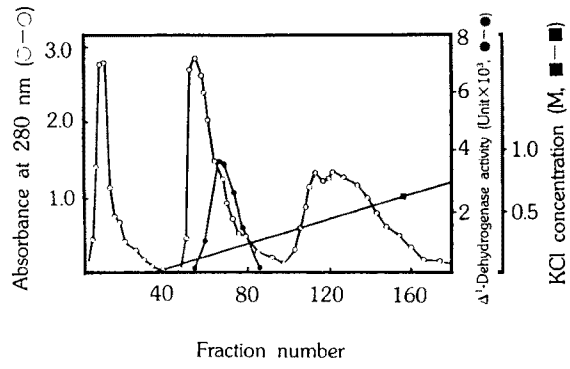

Fig. 2. Chromatography of crude enzyme on DEAE-cellulose column.
Enzyme was eluted with gradient of 20 mM-1 M KCl in 10 mM phosphate (pH 7.5) containing 20% glycerol, at a flow rate of 40 ml/h, and the volume of each fraction was 8 ml.

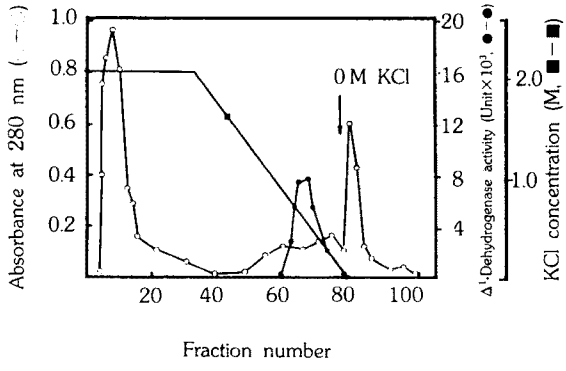

Fig. 3. Hydrophobic chromatography of active fractions from DEAE-cellulose column on phenyl-sepharose column.
Enzyme was eluted with decreasing gradient of 2-0 M KCl in the same buffer of Fig. 2, at a flow rate of 20 ml/h, the volume of a fraction was 7 ml.

Table 2. Summary of the purification procedures of steroid Δ^1-dehydrogenase from hydrocortisone-induced *Arthrobacter simplex* cells of 50 g wet weight

Purification steps	Total activity ($U\times10^3$)	Total protein (mg)	Specific activity ($U\times10^3$/mg)	Yield (%)	Purification fold
100,000×*g* supernatant	6,740	1,171	5.76	100	1.00
Streptomycin sulfate supernatant	6,201	1,147	5.41	92	0.94
DEAE-cellulose	4,651	422.8	11.00	69	1.91
Phenyl-sepharose	2,561	142.5	17.97	38	3.12
Testosterone-agarose	876	1.24	706.45	13	122.65

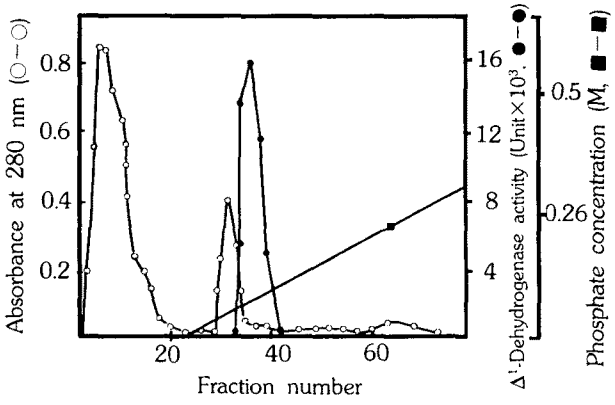

Fig. 4. Affinity chromatography of active fractions from phenyl-sepharose column on testosterone-agarose column.

Enzyme was eluted with 10-500 mM phosphate at a flow rate of 10 ml/h, and the volume of a fraction was 5 ml.

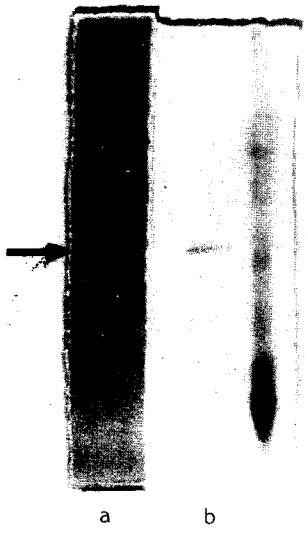

Fig. 5. Non-denaturing PAGE of the purified steroid Δ¹-dehydrogenase.

(a) A band showing enzyme activity stained with nitroblue tetrazolium in the reaction buffer (pH 9.0) containing hydrocortisone and phenazine methosulfate, (b) Coomassie Brilliant Blue stained band.

Determination of the Molecular Weight of the Enzyme

The molecular weight of the native enzyme was estimated to be 100,000 by gel filtration column chromatography on Sephacryl S-200 (Fig. 6). SDS-PAGE showed a single band (Fig. 7), and the molecular weight of the denatured protein was estimated to be 98,000 (Fig. 8). The results from gel filtration chromatography and SDS-PAGE reveal that the steroid Δ¹-dehydrogenase molecule consists of a single subunit.

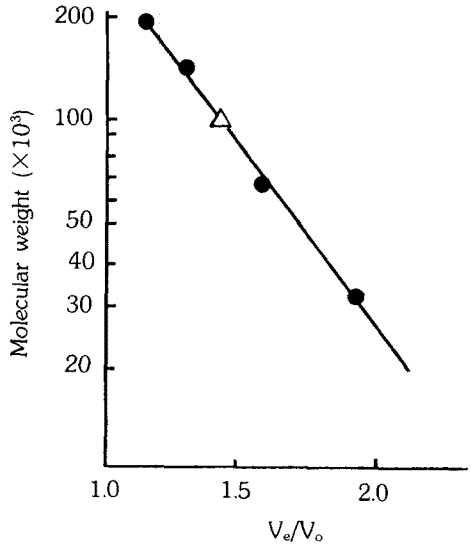

Fig. 6. Molecular weight estimation of the native enzyme by gel filtration column chromatography on Sephacryl S-200 (△, MW 100,000).

Protein markers of known molecular weight (●) were β-amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), and carbonic anhydrase (29,000).

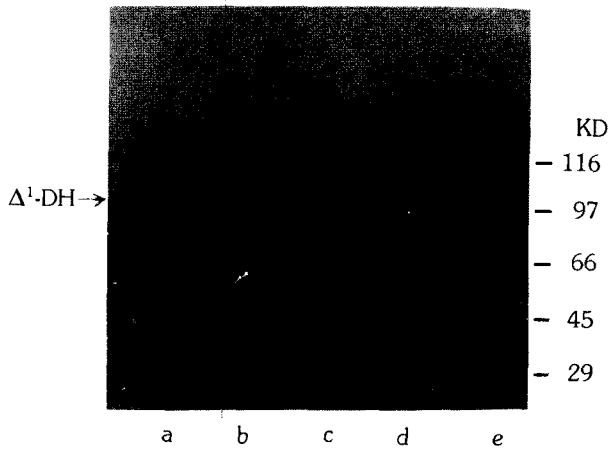

Fig. 7. SDS-PAGE of the enzyme in each purification steps.

Active fractions of (a) DEAE-cellulose column, (b) phenyl-sepharose column, (c) testosterone-agarose column; (d) pooled fractions without enzyme activity of testosterone-agarose column; (e) molecular weight markers (β-galactosidase, phosphorylase b, bovine albumin, and carbonic anhydrase).

Effects of the Artificial Electron Acceptors

The purified enzyme revealed no activity without addition of artificial electron acceptor. Several kinds of electron acceptors were added to the reaction mixture. Phenazine methosulfate (PMS) and 2,6-dichlorophenol indophenol (DCPIP) acted as effective electron acceptors

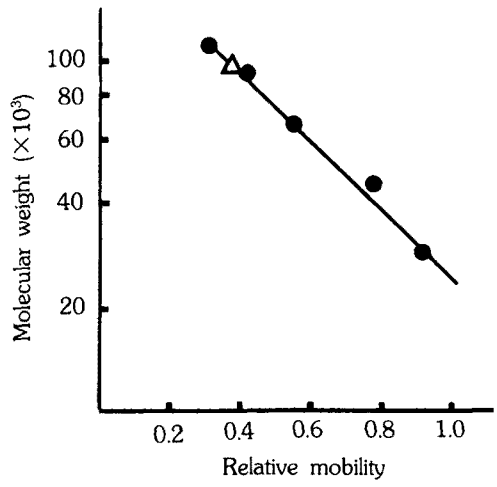

Fig. 8. Molecular weight estimation of the purified steroid Δ^1-dehydrogenase by SDS-PAGE (△, MW 98,000). Protein markers (●) were β-galactosidase (116,000), phosphorylase b (97,400), bovine albumin (66,000), egg albumin (45,000) and carbonic anhydrase (29,000).

Table 3. Effects of artificial electron acceptors on steroid Δ^1-dehydrogenase activity

Electron acceptors	Concentrations (μM)	Enzyme activity (U×10³/10 μl*)	Relative activity (%)
No electron acceptor		0	0
Phenazine	125	222	98
methosulfate(PMS)	250	227	100
2,6-dichlorophenol	125	158	71
indophenol(DCPIP)	250	164	72
NAD^+	125	0	0
FAD	125	0	0
	250	0	0

*10 μl of purified enzyme solution

(Table 3). At the same concentrations, DCPIP showed lower efficiency than PMS. Its efficiency was about 70% of that of PMS.

Subcellular Distribution of the Enzyme

Bacterial cell membrane and cytosol fractions were separated by ultracentrifugation. Comparison of enzyme activities between pelleted membrane and cytosol fractions suggests that the enzyme may be a weakly attached peripheral membrane protein *in vivo* (Table 4). In the presence of artificial electron acceptor, the enzyme activity was distributed evenly in both membrane and cytosol fractions. But in the absence of PMS the enzyme activity was found in membrane fraction only. Because the purified enzyme revealed no activity when electron acceptor was absent (Table 3), the enzyme activity of the membrane fraction may be due to the natural electron accepter(s) in cytoplasmic membrane, indicating that the enzyme is an membrane protein *in vivo*.

DISCUSSION

Since the first recognition of steroid Δ^1-dehydrogenase activity (5) there has been attempts to elucidate the nature of the enzyme in many microorganisms such as *Pseudomonas* (16), *Nocardia* (6), *Bacillus* (10), and *Septomyxa* (1), with crude extracts or partially purified enzymes. But successful solubilization and purification could not be achieved. Only recently, for the first time, 3-ketosteroid-Δ^1-dehydrogenase has been purified and characterized from *Nocardia corallina* (8).

In this study a steroid Δ^1-dehydrogenase was purified from hydrocortisone-induced *Arthrobacter simplex* cells and characterized. The ability as an effective biotransformer of hydrocortisone into prednisolone makes *A. simplex* valuable. Because no information is available whether the only one kind of steroid Δ^1-dehydrogenase occurs in a microorganism after being induced with various kinds of steroids, the microorganism was induced with hydrocortisone and the enzyme was assayed with hydrocortisone as substrate during the purification procedures. Dye reducing assay method was avoided, because unwanted other dehydrogenations could occur simultaneously. Instead of indophenol reduction, the amount of the reaction product was measured directly on the silica gel plates. Although the enzyme possibly be a membrane protein, enzyme activity appeared in 100,000×*g* supernatant when the cells were disrupted with vigorous agitation with glass beads.

Steroid Δ^1-dehydrogenase was purified 123-fold in three steps with 13% yield. The last step of testosterone-

Table 4. Subcellular distribution of steroid Δ^1-dehydrogenase activities in *Arthrobacter simplex* cells

Fractions	With PMS as an electron acceptor		Without artificial electron acceptor	
	Conversion rate of HC* into PDL** (%)	Relative activity	Conversion rate of HC into PDL (%)	Relative activity
Membrane (100,000×*g* pellet)	80	1.00	16	0.20
Cytosol (100,000×*g* supernatant)	87	0.92	0	0.00

*Hydrocortisone, **Prednisolone

agarose affinity column chromatography decisively contributed to the successful purification. Affinity chromatography on adsorbants containing steroidal substrate analog covalently linked to agarose is an extremely valuable technique for the purification of steroid transforming enzymes. *Pseudomonas* Δ^5-3-ketosteroid isomerase, 3α, 3β, and 17β-hydroxysteroid dehydrogenase were successfully isolated from each other and cellular proteins by chromatography on nortestosterone-agarose (3), or on NAD-hexane-agarose (24).

The purity of the enzyme was confirmed by native PAGE and SDS-PAGE, both showing only a single band. Activity staining on native gel also identified the purification of steroid Δ^1-dehydrogenase. The molecular weight of the purified steroid Δ^1-dehydrogenase from *Arthrobacter simplex* was estimated to be 98,000 by SDS-PAGE, and 100,000 by gel filtration. Data from gel filtration and SDS-PAGE indicate that the native enzyme consists of a single subunit. The molecular weight of steroid Δ^1-dehydrogenase from *A. simplex* shows much difference with that of *N. corallina* of 60,500.

The result of the centrifugation experiment suggests that the enzyme is a weakly attached peripheral protein. Without any additional treatments such as detergent or high salt concentration, enzyme activity was found in 100,000×*g* supernatant and the ratio of activities in membrane/cytosol was nearly 1. This contrasts with the *Pseudomonas* enzyme which was completely precipitated with centrifugation at 10,500×*g* for 30 minutes (16). There have been controversial reports about the location of this enzyme *in vivo*. In *Pseudomonas*, it is believed that the enzyme is membrane protein like other steroid transforming enzymes such as 3β, and 17β-hydroxysteroid dehydrogenase (14, 28). The studies in *Arthrobacter globiformis* also indicate that the enzyme is membraneous(19). In *Mycobacterium* a coexistance of two different enzymes of membraneous and of cytosolic was reported (29).

Studies on the enzymatic properties of the purified enzyme will be published. Further studies on the steroid Δ^1-dehydrogenase from *Arthrobacter simplex* may contribute to the improved understanding of steroid-protein interaction and more efficient production of corticosteroid drugs with enhanced activities.

Acknowledgement

This study was supported by a research grant (89-0504-01) from Korea Science and Engineering Foundation, for which authors are very grateful.

REFERENCES

1. **Abul-Hajj, Y.J.** 1972. Stereochemistry of C-1,2-dehydrogenation of 5β-pregnane-3,11,20-trione by *Septomyxa affinis*. *J. Biol. Chem.* **247**: 686-691.
2. **Bae, M., Y.J. Oh, T.G. Min, and M.K. Lee.** 1991. The induction of steroid Δ^1-dehydrogenase from *Arthrobacter simplex* IAM 1660. *Kor. J. Appl. Microbiol.* **19**: 242-247.
3. **Benson, A.M., A.J. Suruda, E.R. Barrack, and P. Talalay.** 1974. Steroid transforming enzymes, p. 557-566. *In* W.B. Jakoby and M. Wilchek (ed.), *Methods in Enzymology* Vol. 34, Academic Press Inc., New York.
4. **Constantinides, A.** 1980. Steroid transformation at high substrate concentrations using immobilized *Corynebacterium simplex* cells. *Biotech. Bioeng.* **22**: 119-136.
5. **Fried, J., R.W. Thoma, and A. Klingsberg.** 1953. Oxidation of steroids by microorganisms. III. Side chain degradation, ring D cleavage and degradation in ring A. *J. Am. Chem. Soc.* **75**: 5764-5765.
6. **Groh, H., R. Komel, V. Deppmeyer, W. Schade, and C. Horhold.** 1980. Steroid transforming enzymes from microorganisms: The reverse reaction of the steroid-1-dehydrogenase from *Nocardia*. *J. Steroid Biochem.* **13**: 1413-1415.
7. **Heeb, M.J. and O. Gabriel.** 1984. Enzyme localization in gels, p. 416-439. *In* W.B. Jakoby (ed.), *Methods in Enzymology* , Vol. 104, Academic Press Inc., New York.
8. **Itagaki, E., T. Wakabayash, and T. Hatta.** 1990. Purification and characterization of 3-ketosteroid-Δ^1-dehydrogenase from *Nocardia corallina*. *Biochim. Biophys. Acta.* **1038**: 60-67.
9. **Itagaki, E., T. Hatta, T. Wakabayashi, and K. Suzuki.** 1990. Spectral properties of 3-ketosteroid-Δ^1-dehydrogenase from *Nocardia corallina*. *Biochim. Biophys. Acta.* **1040**: 281-286.
10. **Jerussi, R. and H.J. Ringold.** 1965. The mechanism of the bacterial C-1,2-dehydrogenation of sterols III. Kinetics and isotope effects. *Biochem.* **4**: 2113-2126.
11. **Kloosterman, J. and M.D. Lilly.** 1985. Maintenance and operational stability of immobilized *Arthrobacter simplex* for the Δ^1-dehydrogenation of steroids. *Enz. Microb. Technol.* **7**: 377-381.
12. **Kondo, E. and E. Masuo.** 1961. Pseudocrystallofermentations of steroid. *J. Gen. Appl. Microbiol.* **7**: 113-117.
13. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T. *Nature.* **227**: 680-685.
14. **Lefebvre, Y.A., R. Schults, E.V. Groman, and M. Watanabe.** 1979. Localization of 3β and 17β-hydroxysteroid dehydrogenase in *Pseudomonas testosteroni*. *J. Steroid Biochem.* **10**: 523-528.
15. **Levy, R. and P. Talalay.** 1959. Bacterial oxidation of steroids I. Ring A degradations of intact cells. *J. Biol. Chem.* **234**: 2009-2013.
16. **Levy, H.R. and P. Talalay.** 1959. Bacterial oxidation of

steroids II. Studies on the enzymatic mechanism of ring A degradation. *J. Biol. Chem.* **234**: 2014-2021.

17. **Lowry, O.H., N.J. Rosebrough, A.C. Farr, and R.J. Randall.** 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
18. **Martin, C.K.A.** 1984. Sterols, p. 79-95. *In* H.J. Rehm and G. Reed (ed.), *Biotechnology*, **6a**, Verlag Chemie, Weinheim.
19. **Medentsev, A.G., A.Y. Arinvasarova, K.A. Koshcheyenko, V.K. Akimenko, and G.K. Skryabin.** 1985. Regulation of 3-ketosteroid-1-ene-dehydrogenase activity of *Arthrobacter globiformis* cells by respiratory chain. *J. Steroid Biochem.* **23**: 365-368.
20. **Mosbach, K. and P.O. Larsson.** 1970. Preperation and application of polymer entrappted enzymes and microorganisms in microbial transformation proceeds with special reference to steroid 11β-hydroxylation and Δ^1-dehydrogenation. *Biotech. Bioeng.* **12**: 19-27.
21. **Nagasawa, M., M. Bae, G. Tamura, and K. Arima.** 1969. Microbial transformation of sterols. Part II Cleavage of sterol side chains by microorganisms. *Agric. Biol. Chem.* **33**: 1644-1650.
22. **Nagasawa, M., N. Watanabe, H. Hashiba, M. Murakami, M. Bae, G. Tamura, and K. Arima.** 1970. Microbial transfromation of sterols. Part V Inhibitors of microbial degradation of cholesterol. *Agric. Biol. Chem.* **34**: 838-844.
23. **Ohlson, S., P.O. Larsson, and K. Mosbach.** 1978. Steroid transformation by activated living immobilized *Arthrobacter simplex* cells. *Biotech. Bioeng.* **20**: 1267-1284.
24. **Shikita, M. and P. Talalay.** 1979. Preparation of highly purified 3α- and 3β-hydroxysteroid dehydrogenase from *Pseudomonas* sp. *Anal. Biochem.* **95**: 286-292.
25. **Sih, C.J., H.H. Tai, and Y.Y. Tsong.** 1967. The mechanism of microbial conversion of cholesterol into 17-ketosteroids. *J. Am. Chem. Soc.* **89**: 1957-1958.
26. **Smith, L.L.** 1984. Steroids, p. 31-77. *In* H.J. Rehm and G. Reed (ed.) *Biotechnology*, **6a**, Verlag Chemie, Weinheim.
27. **Watanabe, M., K. Philips, and H. Watanabe.** 1973. Induction of steroid binding activity in *Pseudomonas testosteroni*. *J. Steroid Biochem.* **4**: 623-632.
28. **Watanabe, M. and L. Po.** 1976. Membrane bound 3β and 17β-hydroxysteroid dehydrogenase and its role in steroid transport in membrane veiscle of *Pseudomonas testosteroni*. *J. Steroid Biochem.* **7**: 171-175.
29. **Wovcha, M.G., K.E. Brooks, and L.A. Kominek.** 1979. Evidence for two steroid 1,2-dehydrogenase activities in *Mycobacterium fortuitum*. *Biochim. Biophys. Acta.* **547**: 471-479.

(Received July 9, 1993)